

First field evidence for alloparental egg care in cooperatively breeding fish

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Abstract

Helping behaviour in cooperative breeders has been intensively studied in many animal taxa, including arthropods, birds and mammals. In these highly social systems, helpers typically engage in brood care and the protection of dependent young. Such helping systems also exist in cooperatively breeding cichlid species of Lake Tanganyika. However, breeding in these species happens in clefts, narrow holes or shelters underneath stones. Therefore, direct brood care by breeders and helpers has thus far only been observed under artificial laboratory conditions. Under natural conditions, brood care behaviour has been estimated indirectly by determining the time spent in the breeding chamber, or by the number of visits to the breeding chamber. The reliability of such substitutes needs to be validated, for instance, by demonstrating alloparental egg-care of helpers through direct observations in nature. Here, we describe direct egg care by a male helper of the cooperatively breeding cichlid *Neolamprologus savoryi* in the field. The helper inspected and cleaned the eggs and defended them against predators. By reconstructing the genetic relatedness using microsatellite markers, we show that the helper was the son of the breeding male, but unrelated to the breeding female. The genetic mother of the helper was defending a different territory next to the one where the helper showed alloparental egg care. This indicates that the helper had dispersed inside the male territory to assist another female to care for his half-siblings. These results demonstrate alloparental egg care without reproductive share in a fish species under natural conditions, underlining that helping behaviour in cooperatively breeding fish has a strong non-self-serving component.

Introduction

Cooperative breeding, where a dominant breeding pair is assisted by subordinate individuals to rear their offspring, represents one of the most complex forms of sociality (Skutch, 1935; Taborsky, 1987; Solomon & French, 1997; Field & Leadbeater, 2016). It evolved in a range of animal species, including arthropods, mammals, birds and fishes (reviewed in: Koenig & Dickinson, 2016; Rubenstein & Abbot, 2017). Helping duties in cooperative breeders are highly variable between species, including vigilance behaviour and food provisioning in birds and mammals (Clutton-Brock, 2016) and egg cleaning and fanning, shelter digging, and antipredator defence in fishes (Taborsky, 1994, 2016). Some of these behaviours, like food provisioning and care of foreign eggs or young, can be called altruistic, as they involve immediate fitness costs to the alloparent without immediate fitness benefits (as defined by Taborsky et al., 2016). Other behaviours, such as antipredator defence and territory maintenance (e.g. shelter digging) might additionally have an immediately self-serving component, especially when they are also shown in the absence of dependent young (Brouwer et al., 2005). To understand the evolution of cooperative breeding systems it is important to clarify whether other individuals than the breeders engage in non-immediately-self-serving helping behaviours, which are expected to increase the survival of dependent young and the fitness of breeders. Care for eggs or young can be observed rather easily under natural conditions in birds and mammals. It is, however, difficult to show direct brood care in nature in cooperatively breeding fishes, because these species typically excavate breeding shelters underneath rocks or breed in narrow clefts or holes, where direct brood care by breeders and helpers cannot be observed. Therefore, researchers often use proxies of presumed brood care, like the time spent in the breeding chamber (cf. Balshine et al., 2001; Tanaka et al., 2018b) or changes in behaviour depending on the presence of juveniles (Brouwer et al., 2005; Bruintjes et al., 2013). Some cooperatively breeding fishes are known for having only few juveniles, which is probably due either to small clutch sizes (e.g. Tanaka et al., 2018a), or to high mortality of eggs and juveniles. The latter may be somewhat compensated by parental and alloparental care, for example by removing fungi or bacteria, or by protection from predators (Knouft et al., 2003; Brouwer et al., 2005). If egg care is

provided by helpers, breeders might further benefit from gaining time and energy to invest in other activities. Nevertheless, individuals engaging in egg care accept energetic costs (Taborsky & Grantner, 1998). To the best of our knowledge, removing fungi, bacteria or debris from the eggs have not been shown to provide nutritional benefits in any fish species. Such benefits would accrue when eggs were cannibalised (e.g. Gomagano & Kohda, 2008; Mehlis et al., 2009). This behaviour is punished, however, in cooperatively breeding fishes (Taborsky, 1985; Zöttl et al., 2013).

Until today, helpers engaging in direct egg care have been observed only in the *Neolamprologus pulcher/brichardi* species complex (Duftner et al., 2007) under laboratory settings (Taborsky, 1984, 1985; von Siemens, 1990; Zöttl et al., 2013). Evidence for such behaviour from the field is hitherto missing for any cooperatively breeding fish species. Here we provide the first evidence of alloparental egg care of a helper in the cooperatively breeding cichlid *Neolamprologus savoryi* (Heg et al., 2005; Garvy et al., 2015) in nature. We furthermore describe the spawning behaviour of this species and apply genetic methods to elucidate the relatedness between different territory members and the brood caring helper.

Methods

Study species

Neolamprologus savoryi is a cooperatively breeding cichlid fish endemic to Lake Tanganyika, East Africa (Heg et al., 2005). Breeding groups are composed of a dominant male and one to several breeding females (Heg et al., 2005; Garvy et al., 2015). Females defend distinct sub-territories, in which they tolerate subordinate individuals of varying age, size and sex. Breeding groups cluster into colonies, and each group defends the territory against conspecific and heterospecific intruders and neighbours (Heg et al., 2008). Subordinates help in territory maintenance and defence (Heg et al., 2005). Furthermore, they have been assumed to help in guarding and cleaning the eggs.

Study site and observation period

Data were collected on 24 September 2016 at Kasakalawe point at the southern tip of Lake Tanganyika, Zambia. The study site was a sandy area at a depth of 10.2 m. Small groups of rocks of sizes between

10 and 40 cm in diameter served as shelter for the fishes. We established a 10 x 10 m grid subdivided into 1 m² covering the whole focal colony. This grid allowed us to draw a detailed map of the habitat inside the colony. The territory borders of the focal groups were determined by 20 min observations a few days prior to the occurrence of the spawning and egg laying and plotted on the map. Based on these territory borders and behavioural observations we marked all potential male and female territories with numbered stones. Our focal group of *N. savoryi* was part of a colony containing 22 dominant males, each defending a territory containing 0 - 5 females (median = 3) and tolerating between 0 and 3 large subordinate males (N = 13) in their territory (median = 0). The breeding females' groups (N = 59) contained 0 to 3 helpers larger than 1.5 cm standard length (median = 1).

Observations and data acquisition

While conducting an experiment in the colony (Josi et al., in prep.), we haphazardly witnessed intense courtship behaviour in one of our focal territories. Spawning took place in this territory at an easily observable position, allowing us to record courtship, spawning and egg care. In total, we recorded 30 min and 16 sec of spawning behaviour. Recordings of egg care started directly after the spawning and lasted for approximately two hours. Within this timeframe, we produced 3 video recordings (1: 13 min 13 sec; 2: 22 min 29 sec; 3: 35 min 30 sec). Video material was afterwards processed with Adobe premiere pro CC and analysed for behavioural frequencies of the breeder male and female, and the helper.

Subsequently we caught all fish of the focal male's territory (i.e., one male, 4 females, 1 helper; see Fig. 1). Standard length (SL) was measured from the tip of the mouth to the posterior end of the vertebral column with an accuracy of ± 1 mm using a 1mm measuring board. Further, the sex was confirmed by external examination of the genital papillae. Finally, we removed a small piece of tissue from the fin for genetic analyses. Afterwards, all individuals were released back to their shelter. They recovered within a few minutes.

Genetic relatedness analysis

To scrutinize the genetic relatedness of the group members, total DNA was extracted from the ethanol preserved fin-clip samples using a magnetic separation protocol (MagneSil™ Paramagnetic Particles, Promega; Kotrschal et al., 2012). Fourteen polymorphic microsatellite loci were used to determine relatedness (loci UNH154, UNH106 (Lee & Kocher, 1996); NP007, NP773, ULI2 (Schliewen et al., 2001); Pzeb3, Pzeb4 (Van Oppen et al., 1997); TmoM11, TmoM13, TmoM25, TmoM27 (Zardoya et al., 1996); UME003 (Parker & Kornfield, 1996); UNH1009 (Carleton et al., 2002), and Ppun21 (Taylor et al., 2002). Some of these sequences were already optimized for the closely related species *N. pulcher*. DNA was amplified using the QIAGEN® Multiplex PCR Kit (Qiagen), allowing co-amplification of several locus-specific, fluorescently labelled primer pairs in one single PCR reaction. We used two different primer sets containing seven primer pairs each to amplify the 14 microsatellite markers. PCR reactions were attained in a 10 µl volume containing 1 µl of the genomic DNA, 5 µl 2x QIAGEN Multiplex PCR Master Mix, 3 µl H₂O and 1 µl of 10 x primer mix consisting of fluorescently labelled forward and non-labelled reverse primer pairs with end concentrations of 0.4 to 0.6 µM each, according to the intensity of the respective amplification products. The fluorescent dyes were: 6-FAM (blue), HEX (green), Yakima Yellow (green), ATTO550 (yellow), ATTO565 (red) (Microsynth), VIC (green) and PET (red) (Thermo Fisher). Amplification was performed in a GeneAmp® 9700 PCR System (Applied Biosystems) using the following cycling parameters: 15 min at 95°C, 35 cycles at 95°C for 30 s, 57°C for 3 min and 72°C for 60 s followed by a final elongation step of 72°C for 15 min. Fluorescent PCR fragments were visualized by capillary electrophoresis on an ABI3100® Genetic Analyser (Applied Biosystems). GeneScan 500 LIZ (Thermo Fisher) was used as an internal size standard and the fragments were analysed using the GeneMarker® Analysis software version 2.4.0 (SoftGenetics). We reconstructed relatedness within the focal group using the Simpson-assisted descending ratio algorithm in KINGROUP v2.1 (Konovalov, 2006), compared against the null hypothesis of no relatedness.

Results

Group structure

The breeding male (M1) of the focal group measured 60 mm SL. His territory contained 4 females defending sub-territories (F1: 44 mm; F2: 45 mm; F3: 46 mm; F4: 48 mm; all measures in SL; for home ranges see Fig. 1). Female F4 had a single male helper (H4; 27 mm SL) in her territory. The relatedness analysis revealed that the breeding male was the genetic father of helper H4, while female F2 was its genetic mother ($p < 0.01$, type II error = 0 %). Furthermore, female F3 was either the daughter or sister of the breeding male, while the other females were unrelated to him ($p < 0.01$, type II error = 0 %).

Spawning behaviour

While female F4 showed spawning behaviour with the territory owner, she also showed 32 times pseudo-spawning (behaviourally identical to spawning but without eggs being laid) with a neighbouring male (M2 (61 mm SL); see video supplement material 1). Thus, she switched several times between the pseudo-spawning site and the egg deposition site (see Fig. 1 and video supplement material 1). During pseudo-spawning, female F4 received aggression from the breeding male M1 as well as from female F2 (see video supplement material 1). The male M2 never showed any aggression towards female F4, but observed or inspected her rather closely during pseudo-spawning. Based on the typical male posture and behaviour during the release of sperm, we counted that male M2 released 9 times sperm during pseudo-spawning, while the female did not lay any eggs. At the egg deposition site, she laid eggs that were fertilized directly afterwards by the dominant breeding male M1. During spawning, no other individual beside the breeding male M1 and female F4 approached the egg deposition site. In total, six eggs were deposited which does not seem to be an exceptional clutch size for *N. savoryi*, as during a second observation in another territory a clutch of 10 eggs was recorded (DJ, personal observation). After the spawning, M1 shortly inspected the eggs (0.8 seconds) while M2 never inspected them. However, the breeding female (F4) and her helper (H4) inspected, cleaned, and defended the eggs (see Fig. 2; video supplement material 2). During the 71 min of recordings after spawning had ended, the female showed egg cleaning behaviour six times, defended the spawning site against conspecific and heterospecific intruders 20 times, and inspected the eggs for a total period of 106 seconds. In the same time period, the helper cleaned the eggs 28 times, defended once against a

heterospecific egg predator (*Telmatochromis vittatus*), and inspected the eggs for a total period of 339 seconds. Most defence behaviour was shown by the breeding female against the facultative egg predator *Telmatochromis vittatus* (twice during the spawning and 7 times afterwards), the piscivorous eel *Mastacembelus moorii* (8 times during spawning and once after the spawning), and against conspecifics (5 times during spawning and 12 times afterwards; see Fig. 2 and video recordings in supplement material 1, 2). The breeding male M1 defended the eggs only against conspecific intruders after the spawning (6 times in total), but did not engage in cleaning the eggs.

Discussion

To fully comprehend the occurrence of altruistic behaviour in cooperative breeders it is important to show alloparental care under natural conditions. Here we provide results from the first field observations of egg care behaviour by a helper in a cooperatively breeding fish. The caring helper was the genetic son of the breeding male, whereas it was unrelated to the female laying the eggs. The genetic mother of the helper defended the neighbouring sub-territory (F2) of the egg-laying female (F4; see Fig. 1). This indicates that helpers are tolerated not only in their mothers' territory, but also in other female subgroups of the breeding male. Helpers might hence be recruited from neighbouring subgroups, depending on the need for help. The helper carefully inspected and cleaned the eggs and showed vigilance behaviour close by. This is in accordance with the helping behaviour of *N. pulcher* described from the laboratory (Taborsky, 1984, 1985; von Siemens, 1990; Zöttl et al., 2013). The helper's effort cannot be explained by a share in reproduction, as it was too small to be sexually mature (D. Heg, personal communication) and as it was not close to the egg laying site while spawning took place. Hence, the helper might have gained indirect fitness benefits by caring for his half-siblings (Bruitjes & Taborsky, 2011), and delayed direct benefits through group augmentation (Kokko et al., 2001) by increased egg survival, and/or by being allowed to stay in the female's territory, where it enjoys protection from predation ("pay-to-stay" Taborsky, 1985; Bergmüller & Taborsky, 2005; Zöttl et al., 2013; Fischer et al., 2014). Compared to the breeding female, the helper cleaned the eggs 4.6 times more often and spent 3.2 times more time with inspecting the eggs, whereas the female spent 7 times

more effort in defence against egg predators. These results indicate that breeding females and helpers may specialize in different duties during egg care, suggesting division of labour as demonstrated in the cooperatively breeding congener *N. pulcher* (Bruitjes & Taborsky, 2011).

The clutch had disappeared by the next morning, probably because the egg deposition site was quite exposed to predators. Especially during the night, eggs may be vulnerable to predation by nocturnal predators. Indeed, already during daytime the eel *Mastacembelus moorii* and the facultative egg predator *Telmatochromis vittatus* tried repeatedly to approach the egg deposition site, but they were chased away by the breeding female (see supplement material 1, 2). After the eggs disappeared, the helper was no longer observed at the egg deposition site, indicating that he had no other interests in this particular part of the female's territory.

The spawning was frequently interrupted by pseudo-spawning events. Such pseudo-spawning behaviour has been shown in other cooperatively breeding cichlids as well (Taborsky, 1985). While the function of this behaviour is not fully understood (Kohda, 1995; Heg et al., 2008), it has been interpreted as evidence of mate choice (Egger et al., 2008). Alternatively, it might serve to coordinate the behaviour of the spawning partners. Our observation might indicate that pseudo-spawning of the female can also serve to reduce reproductive conflict through paternity insurance between breeding males and the female. The female showed pseudo-spawning behaviour with the neighbouring male at a different location than the egg deposition site. Additionally, the neighbouring male released sperm at the pseudo-spawning site and afterwards never visited or inspected the egg deposition site. However, whether such behaviour leads to a reduction of disturbances during the actual spawning needs to be experimentally tested in future studies.

In summary, we observed for the first time direct alloparental egg care behaviour in a cooperatively breeding fish in the field. These observations may enhance our appreciation of the evolutionary mechanisms underlying cooperative breeding in fishes and in general.

Author contributions

212 DJ, MT and JGF conceived the study; MT and JGF organized funding; DJ conducted fieldwork, prepared
213 the video material and conducted the genetic analyses; DJ wrote the first draft of the manuscript,
214 which was edited by MT and JGF; all authors approved the final version of the manuscript

215 **Conflict of interest**

216 The authors declare no conflict of interest.

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226 **Ethical Note**

227 Data collection caused minimal disturbance to the animals and followed the regulations of the Zambian
228 Prevention of Cruelty to Animals act.

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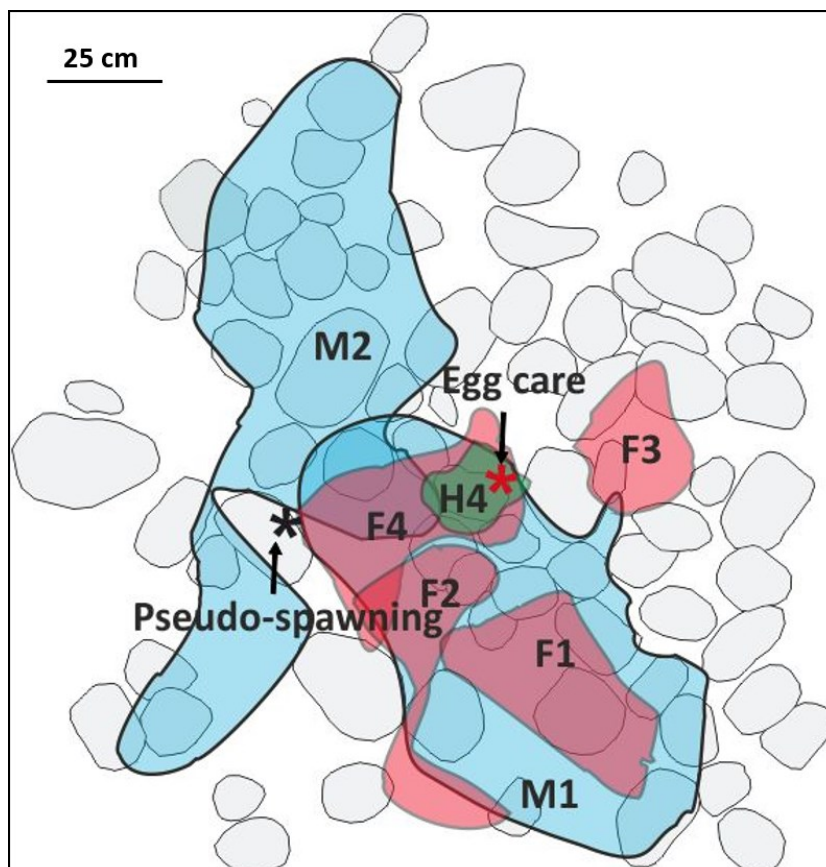
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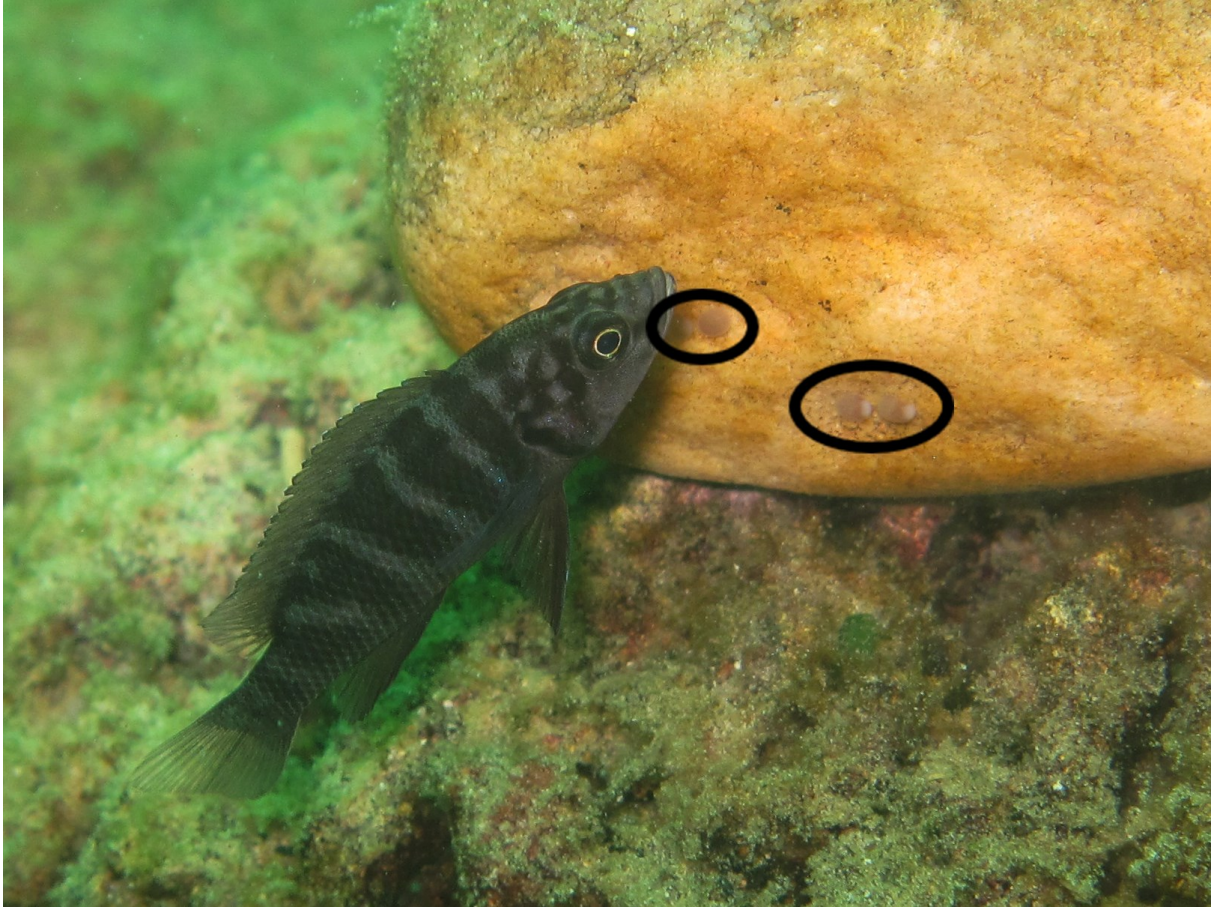
Figure 1

Home ranges of the fish observed in this study. Shown are territories of two neighbouring males (M1, M2). M1 guarded four breeding females (F1 - F4) in his territory, and M2 monopolized three breeding females (not indicated in the map). Female F4 had 1 male helper. The location of the egg deposition site (red star) and the pseudo-spawning site (black star) are indicated. Grey structures indicate individual rocks.



360 **Figure 2**

361 Four out of six eggs (two eggs per black circle) laid by the breeding female and inspected by the helper
362 (H4; 27 mm). The egg deposition site was on one of the stones used for marking the different
363 territories.



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